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Ammonia Pulses and Metabolic Oscillations Guide Yeast Colony Development

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On solid substrate, growing yeast colonies alternately acidify and alkalinize the medium. Using morphological, cytochemical, genetic, and DNA microarray approaches, we characterized six temporal steps in the "acid-to-alkali" colony transition. This transition is connected with the production of volatile ammonia acting as starvation signal between colonies. We present evidence that the three membrane proteins Ato1p, Ato2p, and Ato3p, members of the YaaH family, are involved in ammonia production in *Saccharomyces cerevisiae* colonies. The acid-to-alkali transition is connected with decrease of mitochondrial oxidative catabolism and by peroxisome activation, which in parallel with activation of biosynthetic pathways contribute to decrease the general stress level in colonies. These metabolic features characterize a novel survival strategy used by yeast under starvation conditions prevalent in nature.

INTRODUCTION

Most of our knowledge on unicellular organisms originates from studies on microbial cultures growing in liquid media. In nature, microorganisms hardly ever grow exponentially even although this is usual in laboratory conditions. In contrast, "natural" unicellular microorganisms often create multicellular communities attached to solid surfaces (e.g., fruiting bodies, colonies, and biofilms). Meunier and Choder (1999) showed that the growth of a solitary *Saccharomyces cerevisiae* colony is biphasic, starting with a "rapid growth phase" (24 divisions approx.) similar to the exponential growth phase in liquid culture. This phase is followed by a "slower growth phase," during which the cells in the center of a colony gradually enter the stationary phase and growth continues predominantly on the periphery. Scanning electron microscopy revealed that in young *S. cerevisiae* colonies the cells divide randomly and their density is relatively low. At later stages, the cell divisions become more oriented and density increases (Varon and Choder, 2000). Reynolds and Fink (2001) reported *S. cerevisiae* ability to attach to the cell solid surfaces. This could be the initial step in biofilm formation, a process that was believed to be restricted to "wild" yeasts, particularly *Candida* sp.

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Our previous study revealed that individual colonies exhibit a periodical behavior, changing the pH of their surroundings from acid to nearly alkali and vice versa (detailed profile of ammonia pulses of *S. cerevisiae* colonies used in this study is at http://www.biologie.ens.fr/fr/genetiqu/puces/publications/ATO/index.html, Figure 7S). In the "acid" phase the colonies are growing, whereas in the "alkali" phase growth is transiently inhibited. This behavior is widespread throughout different yeast species. The ammonia released during the alkali phase acts as a long-range signal between neighboring colonies, influencing their "acid/alkali" periodicity and their growth (Palková *et al.*, 1997). Colonies exposed to volatile ammonia respond by enhancing their own ammonia production, regardless of their current developmental phase. Ammonia production in neighboring colonies is thus amplified for a few hours, especially in their mutually adjacent regions. As a result, the growth of neighboring colonies becomes concurrently inhibited by an unknown mechanism. Subsequently, ammonia production gradually declines, and colonies start to grow again and consequently enter the next acid period. This induced ammonia production synchronizes the acid/alkali pulses in neighboring colonies and directs their growth to the free space. Moreover, in *Candida mogii* colonies, ammonia induction is accompanied by conspicuous changes in colony and cell morphology, suggesting that the colony actively responds to ammonia. (Palková and Forstová, 2000). Mutant analysis revealed a connection between the uptake of external amino acids and the ability of yeast colonies to produce ammonia. Amino acid permease mutant colonies exhibit

decreased ammonia production. In contrast, changes in external ammonium (NH_4^+) concentration and defects in the Mep ammonium permeases do not influence the ammonia-mediated signaling (Zikánová *et al.*, 2002).

All these observations raise several fundamental questions. First, why and how do colonies periodically switch from an acid growth phase to the ammonia-producing period (alkali phase) and what determines the timing of the transition? Second, what is the mechanism of NH_3 release and "sensing" that induces and enhances ammonia production in neighboring colonies, regardless of their current phase? To study these processes, we performed genome-wide analyses of gene expression changes in *S. cerevisiae* colonies in different phases of their development. This, together with analyses of specific yeast mutants, allowed us to set up an overview of metabolic changes occurring within *S. cerevisiae* colonies during their acid/alkali transition. We also identified new proteins putatively involved in ammonium export and obtained indications that the transition to the ammonia-producing phase is important for long-term colony survival. Genome-wide study of gene-expression in yeast colonies taken directly from solid media allowed us to unravel for the first time the metabolic pathways used for survival under starvation conditions on solid support such as those encountered by yeast in nature.

MATERIALS AND METHODS

Strains and Media

Strain *S. cerevisiae* BY4742 (MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0) and all isogenic mutants were from the EUROSCARF collection. Colonies were grown on GM agar (1% yeast extract, 3% glycerol, 2% agar, 30 mM $CaCl_2$) or GM-BKP agar (GM, 0.01% bromcresol purple).

Ammonia Production Measurement

Ammonia released by growing colonies was absorbed into acidic traps as described (Palková *et al.*, 1997, www.natur.cuni.cz/~zdenap) at the intervals indicated in Figure 3B. Alternatively, cells picked from colonies were suspended in 10 mM MES (1×10^9 cells·ml^{-1}) and incubated on roller. Individual samples were taken in indicated times (see Figure 4, E and F) and centrifuged. The amount of ammonia in various liquid samples was determined by use of the Nessler reagent.

Methyl Ammonium Resistance

Cells picked from either acid or alkali colonies were treated with various concentrations of methylammonium (0–100 mM) for 1 h. Cells were subsequently plated onto YPD plates at different concentrations and the number of surviving cells (colony forming units) was determined.

Neutral Red Staining

Cells picked from colonies in particular phases were stained with 1 mg/ml neutral red dye and immediately observed by microscopy.

Isolation of Amino Acids and HPLC Analysis

For total intracellular amino acids isolation, two or three colonies were suspended in 1.2 ml of MES Cu^{2+} buffer (5 mM MES, 0.4 mM $CuCl_2$, pH 6). One milliliter of the suspension was transferred to a tube with 2 ml of deionized water, boiled for 15 min, cooled, and centrifuged. The supernatant was filtered through a nylon membrane filter (0.22 μm pore size) and stored at −20°C. Alternatively, two or three colonies were suspended in 1.2 ml of MES Cu^{2+} buffer, and cytosolic and vacuolar amino acids were extracted following the method of Ohsumi *et al.* (1988) modified by Gent and Slaughter (1998). The concentration of amino acids was determined by liquid chromatography with precolumn derivatization using 6-aminoquinolyl-*N*-hydroxysuccinimidyl and fluorescent detection.

RNA Isolation and Northern Analysis

For total RNA isolation, colonies (approximately 10^{10} cells) were directly suspended in TES buffer (10 mM Tris, pH 7.5, 10 mM EDTA, 0.5% SDS). The exact procedure is at www.biologie.ens.fr/fr/genetiqu/puces/protocoles_puces.html. The RNA samples were quantified on spectrophotometer. For Northern blot, 15 μg of total RNA was loaded. The rRNA content was visualized by EtBr staining and used as a reference for normalization of the signals.

Microarray Analyses

Microarray slides containing most of the yeast open reading frames (5885 PCR products) were obtained from Hitachi Software and DNAChip Research, Inc. Two micrograms of mRNA were used for each reverse transcription reaction. Detailed protocols are at www.biologie.ens.fr/fr/genetiqu/puces/protocoles puces.html. The arrays were read by a genepix 4000 scanner (Axon Instruments, Union City, CA) and were analyzed with the genepix 3.0 software. For the microarray/kinetic experiments, time point 2 was used as the cohybridization reference sample for analyzing gene expression in phase 1, 3, 4, 5, and 6. For convenient visualization of the expression profiles, the data were mathematically transformed, so that phase 1 was the numerical reference for cluster representation (see Figure 2). Each microarray result presented here is an average of at least 10 independent biological measurements.

Biocomputational Analyses of Microarray Data

We excluded artifactual spots, saturated spots, and low signal spots. On average, 85% of the spots on each microarray used in this study were significantly quantified (minimum 74%, maximum 91%). Assuming that most of the genes have unchanged expression, the Cy3/Cy5 ratios were normalized by use of the median of all the ratios for each experiment using the Arrayplot software (Marc and Jacq, 2002; available at www.biologie.ens.fr/yeast-publi.html). We considered than the expression of a gene was reproducibly changed either when the average ratio was >2 and varied by <15% between replicated experiments (which means that a gene with an average value of 2 was never measured <1.7) or when the measured ratio was always >3. We clustered the data from the kinetic experiments with the "make tree" module of Jexpress (Dysvik and Jonassen, 2001). The cluster shown in Figure 3 was generated by Treeview (Eisen *et al.*, 1998). To search for consensus sequences in the promoters (between −800 and +1), we used the Consensus module of RSA tools (van Helden *et al.*, 2000).

Protein Sequence Analyses

Protein sequence analyses were performed with WU-Blast2, Clustalw, TMHMM, PROSIT, Bork's alignment tools, Jpred2, and JalView. The protein sequences of *Saccharomyces bayanus*, *Zygosaccharomyces rouxii*, *Saccharomyces sorbitophila*, *Pichia angusta*, and *Kluyreromyces marxianus* were found at the Génolevures, of *Candida albicans* at the "*Candida* Sequencing" page and of *Chlamydomonas* at the TAIR database. The links to the particular web sites are at http://www.biologie.ens.fr/fr/genetiqu/puces/publications/ATO/index.html

RESULTS

Analysis of Distinct Developmental Stages of the Acid/Alkali Transition

To reveal the expected waves of gene expression changes during the acid/alkali transition, we attempted to define six particular steps in acid/alkali transition period of *S. cerevisiae* BY4742 giant colonies (Palková *et al.*, 1997), starting from "fully acid" (phase 1) and finishing in "fully alkali," ammonia producing stage (phase 6) of the colony development. In parallel with the monitoring of pH alterations (Figure 1A), we used two "physiological" markers to follow the transition process: 1) changes in neutral red dye staining of vacuoles of yeast cells taken from colonies occurring in the different phases (Figure 1B) and 2) changes in intracellular amino acid concentrations and distribution between vacuole and cytoplasm (Figure 1, C and D). Intracellular vesicles of cells taken from colonies in the acid developmental phases (1 and 2) were not stained by neutral red (Figure 1B). At the beginning of the transition to the alkali phase ("neutral" phase 3), the first small neutral red stained vesicles appeared, indicating thus that these vesicles were more acidic than the cytoplasm. The number of acidic cellular vesicles increased as the transition progressed, being the most evident in phase 4, when the first visible alkali on colonies appeared. In the later alkali phases (5 and 6), larger red vesicles were observed (Figure 1B). The appearance of neutral red–stained vesicles indicated that the proton gradient between the intracellular vesicles and the cytoplasm had changed during the acid/alkali transition. The intracellular pools of most amino acids decreased in phase 3 and then increased again in later phases (phases 5 and 6; Figure 1C). This concerned mainly vacuolar amino acids; the concentration of cytoplasmic amino acids remained nearly unchanged (Figure 1D; Figure 8S at http://www.biologie.ens.fr/fr/genetiqu/puces/publications/ATO/index.html).

To follow the kinetics of gene expression changes, we isolated total RNA from colonies growing on a solid medium with pH dye indicator (GM-BKP), harvested at the following times of their development (Figure 1A): colonies (1) in "fully acid" phase (7 d), (2) in "late acid" phase (9 d), (3) in "neutral" phase (10 d), (4) in "early alkali" phase (10.5 d), (5) in "developing alkali" phase when the initial violet color appeared between colonies (11 d), and (6) in "fully alkali" phase when an intensive violet color was observed between neighboring colonies (12 d). The RNA samples (phases 1–6) were compared by microarray analysis. The expression of ~200 genes (of 5885 tested) was significantly changed in at least one phase (Figure 2). The changes in expression of selected genes were confirmed by northern hybridization (Figure 1E). In parallel experiments we compared the transcriptomes isolated from the phase 1 acid and phase 6 alkali *S. cerevisiae* monocolonies each arising from a single cell (~50 monocolonies per experiment). The observed changes (unpublished data) were equivalent to those obtained with giant colonies (comparing phase 1 and 6) and confirmed that the acid/alkali pulses in both types of colonies are similar and only their timing differs.

During the investigated period of colony development (7–12 d), the majority of the cells should be either in stationary phase or in slow growth phase, as defined by Meunier and Choder (1999). The feasibility to detect significant changes in expression of specific genes indicates that despite their supposed "quiescent" state, such cells still exhibit an ability to reprogram their metabolism. In contrast to published microarray experiments performed with exponentially growing cells in liquid cultures, observed changes of gene expression in colonies were in lower range, most often up to threefold induction/repression. In this regard, it should be kept in mind that yeast colony (more resembling yeast populations in nature) exhibits internal cell heterogeneity (Mináriková *et al.*, 2001). So far, we are not able to dissect yeast colony and to analyze cell subpopulations at the microarray level. Nevertheless, most of genes induced or repressed during the process of acid/alkali transition belong to distinct, physiologically coherent groups (Figure 2), which seems to attest that most, if not all changes in gene expression concern the major subpopulations of the colony. Of course, we cannot exclude the existence of more than one prominent cell subpopulation determining behavior of multicellular colony, reminiscent of the situation described previously in the developing multicelullar slime mold *Dictyostelium discoideum* (Cotter *et al.*, 1999). Therefore, all changes discussed later relate to the entire colony.

To prove that detected changes in gene expression are involved in ammonia production and/or in the process of acid/alkali transition, we analyzed the colonies of some corresponding gene-deleted strains (*ycr010c*, *ydr384c*, *ynr002c*, *pho89*; Figure 3). We assessed the alkali appearance (violet color of the pH dye indicator in the agar) and ammonia production of the deletion mutants and parental isogenic strain. The violet color appeared in the deletion mutants and parental colonies at the same time (10–11 d); however, it then continued to intensify in only the wild-type colonies (Figure 3A). This suggests that the deletion of the relevant genes results in a defect in ammonia induction. Direct measurement of released ammonia confirmed that the mutant colonies release less ammonia (Figure 3B).

Early Induction of Amino Acid Metabolism: A Prelude to Ammonia Production?

The group of genes activated between phases 1 and 2 includes several genes involved in the biosynthesis/degradation pathways of different amino acids (Figure 2). The product of *GCV1* (2.5-fold induction), glycine decarboxylase, participates in NH_3 release from glycine (McNeil *et al.*, 1996) and may be involved in initiated ammonia production. Another induced gene, the product of which might be directly involved in NH_3 production, is *SRY1* (2.1-fold induction) encoding a protein similar to *Escherichia coli* threonine dehydratase. In contrast to most amino acid metabolic genes, which were induced during the early stages, the *AAT1* gene encoding mitochondrial aspartate aminotransferase, was induced in phase 4 and culminated in phase 5. This indicates another later change in amino acid metabolism, which might lead to the resynthesis of intracellular amino acid stocks after the beginning of ammonia production. This is in agreement with the transient decrease in intracellular amino acid concentrations, followed by their increase almost up to the original level (Figure 1C). Additionally, some of the genes encoding enzymes involved in amino acid degradation were repressed in phase 6. One of them, *CHA1* encoding serine/threonine deaminase (twofold repression) might also be involved in ammonia production.

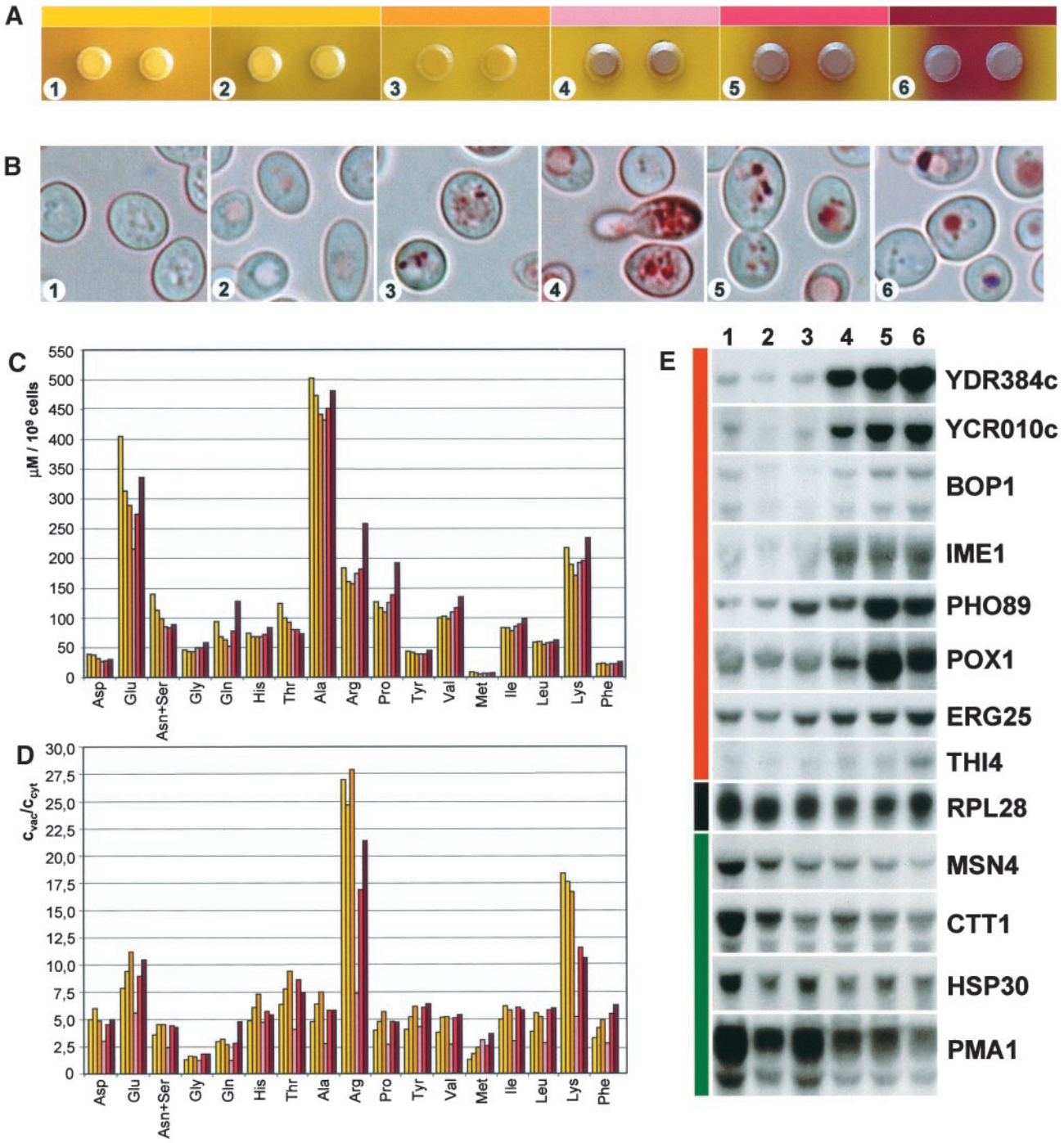

Figure 1. The kinetics of changes occurring in colonies during their acid/alkali transition. (A) Giant colonies of BY4742 growing on GM-BKP. Phases 1 (7 d), 2 (9 d), 3 (10 d), 4 (10.5 d), 5 (11 d) to 6 (12 d) are also indicated by colored rectangles above the photos. (B) Cells exhibiting characteristic neutral red staining in individual phases 1–6. Magnification, ×3000. (C) Changes in total intracellular concentrations of individual amino acids during phases 1–6. Color of the bars corresponds to phases 1–6 as indicated in A. (D) Ratio of the concentrations of individual amino acids in vacuoles and in the cytoplasm during phases 1–6. (E) Northern hybridization of RNAs isolated from colonies occurring in stages 1–6. Probes for genes that were induced in microarray experiment are indicated by a red rectangle, those that were repressed by a green rectangle, and the nonregulated control, *RPL28*, by a black rectangle.

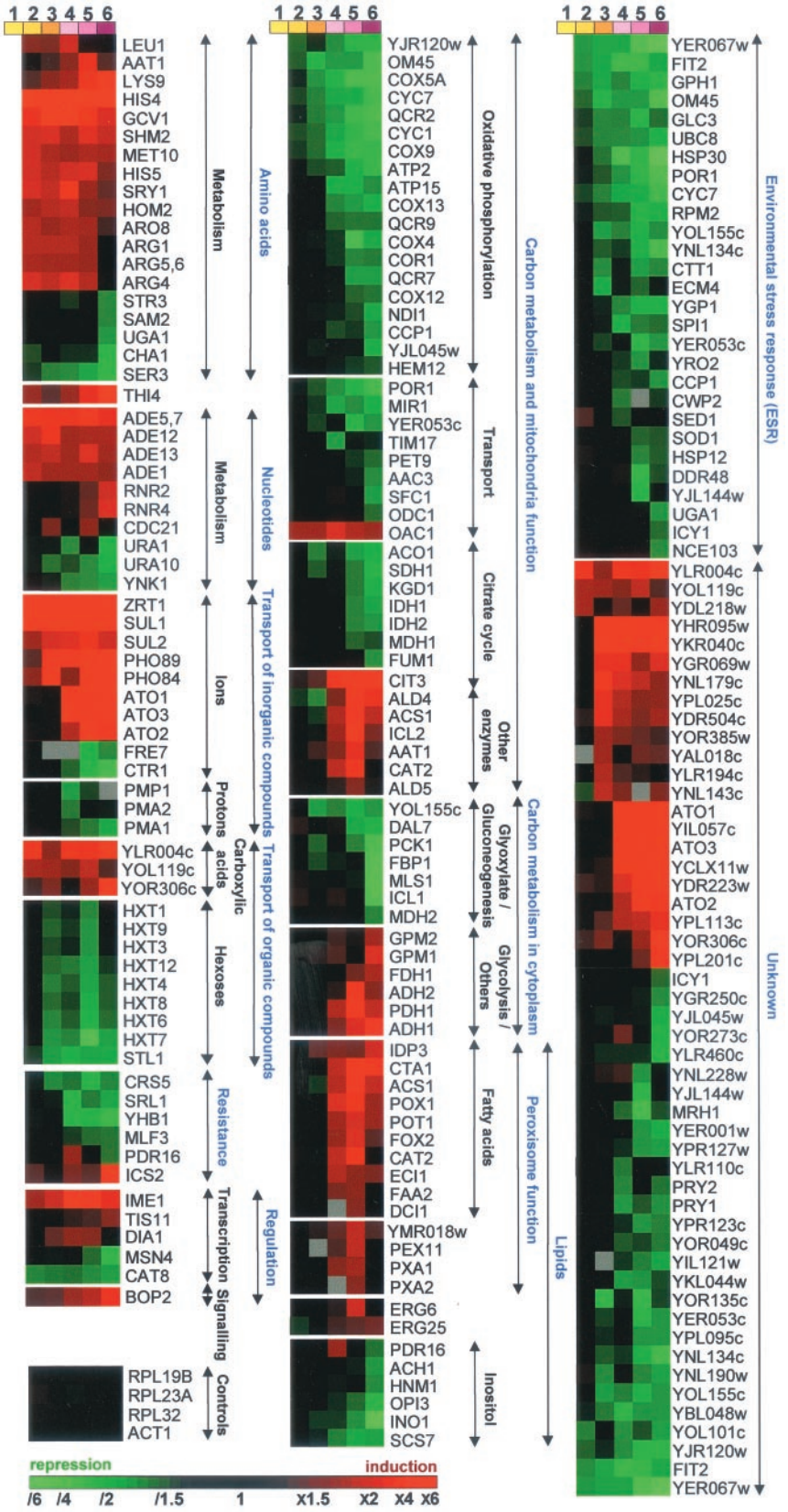

Figure 2. The expression variations in colonies during their acid/alkali transition. The time-course transcription profiles of 200 genes, the expression of which significantly changed in at least one phase during the acid/alkali transition, have been arranged according to their functions. Information obtained from YPD, SGD, YTPdb, MIPS, and the literature cited in the text were used for functional classification. The genes *ATO1 (YCR010c), ATO2 (YNR002c),* and *ATO3 (YDR384c)* were defined in this work (see text and Figure 4). The numbers and color bars at the top of the clusters define the phases. The change in expression is indicated by a colored square (color bar on the bottom right). The corresponding values are on Table 2S at http://www.biologie.ens.fr/fr/genetiqu/puces/publications/ATO/index.html. Three ribosomal protein-coding genes (*RPL*) and the *ACT1* gene for actin represent the nonregulated genes.

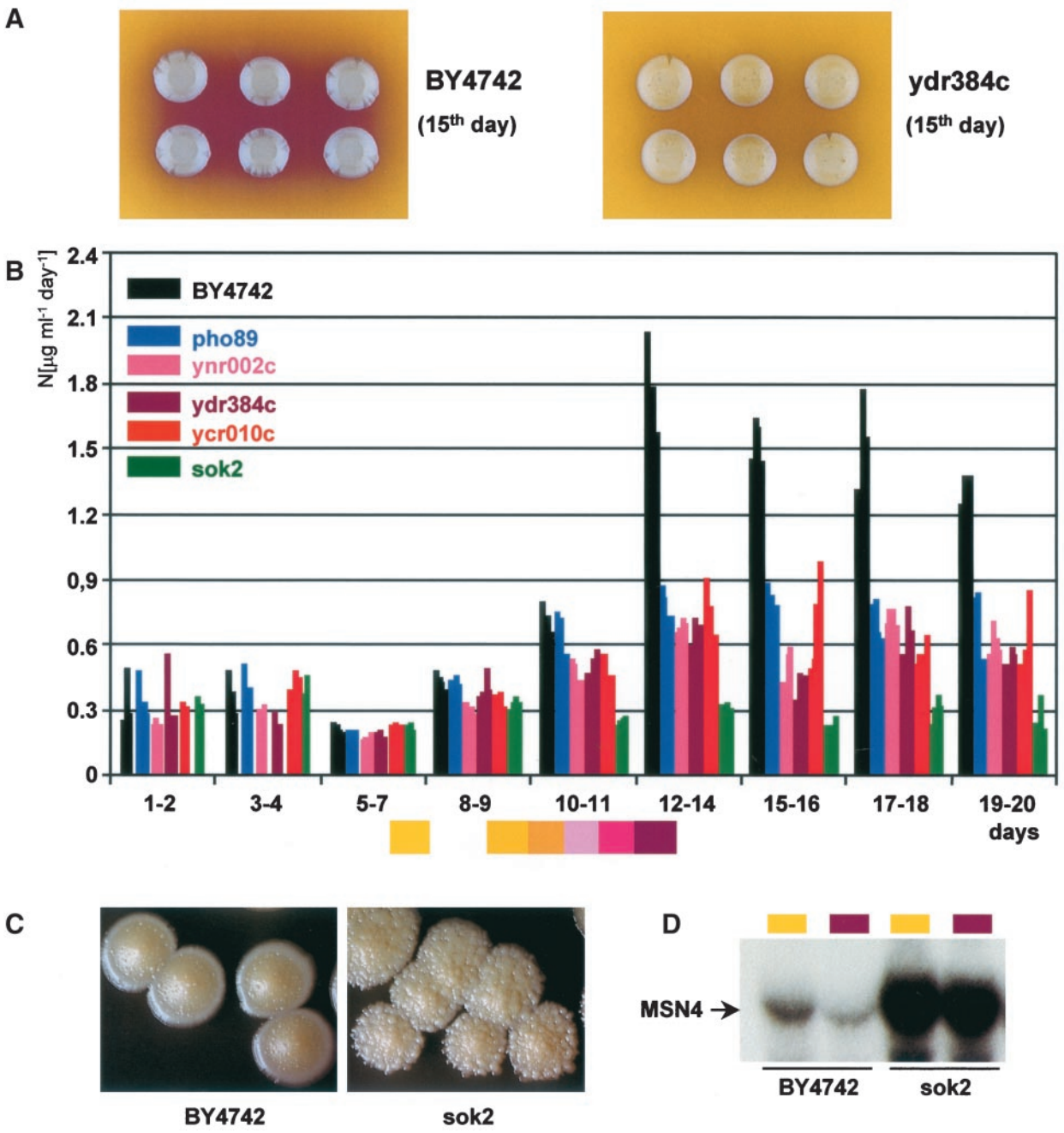

Figure 3. Colonies of mutants in genes induced during the acid/alkali transition exhibit defect in ammonia production. (A) Fifteen-day-old giant colonies of the parental strain BY4742 and of the *ydr384c* mutant growing on GM-BKP. (B) Ammonia production by giant colonies of BY4742 and *pho89*, *ynr002c*, *ydr384c*, *ycr010c*, and *sok2* mutants. The ammonia produced by colonies growing on GM agar was absorbed (during indicated intervals) and measured as described. Color scale indicates visible acid/alkali transition of the parental strain. (C) Morphology of 20-d-old monocolonies of BY4742 and the *sok2* growing on GM agar. (D) Northern analysis of *MSN4* gene expression in giant colonies of BY4742 and the *sok2*. RNA was isolated from colonies either in the acid phase (9-d-old, yellow rectangle) or in the alkali phase (13-d-old, violet rectangle).

A Role for the Ato Proteins in Ammonia Production by Yeast Colonies?

Some of the most strongly and quickly induced genes during the first 12 h of ammonia production (between phases 3 and 4, Figure 2) are three genes encoding *S. cerevisiae* members of the YaaH family (TC 9.B.33, see http://www.biology.ucsd.edu/~msaier/transport/). Two of them, Ynr002p and Ycr010p, display strong amino acid similarity (78%) to each other. The third one, Ydr384p, is less homologous (35%).

Single mutants in any of these three genes are viable and do not exhibit major phenotypes in liquid media (see YPD). Their protein products, with unknown function, have six predicted transmembrane domains (http://www.cbs.dtu.dk/services/TMHMM-2.0/). The Ycr010p exhibits a weak homology with putative ammonium transporters from *Caenorhabditis elegans* (P54145, T15413).

We used the Blast search and Clustal alignment to identify and compare the protein sequences of 17 new members of the YaaH family from different species of bacteria, archaea, yeast, and other eukaryotes (*Leishmania*, *Chlamydomonas*). These proteins share three conserved domains, the second of which mapped to the region of homology of the Ycr010p with the *C. elegans* putative ammonium transporters (Figure 4A). In this homologous region of *C. elegans* proteins PROSIT identified a 25-amino acid sequence with an "ammonium transporter signature" (ATS; Figure 4B). Within this region, the amino acids Phe and Trp at positions 14 and 15 are conserved in all identified proteins (Figure 4B). According to the TMHMM prediction, the region homologous to the *C. elegans* ATS is located partially on the internal loop and partially on adjacent transmembrane span 3 in most of the members of the family (Figure 4C). The average distance tree revealed two main phylogenetic clusters as well as an additional more distant sequence from *Chlamydomonas* (Figure 4D).

The ammonia production by *S. cerevisiae* strains individually deleted in *YCR010c*, *YDR384c* and *YNR002c* genes revealed that colonies of all three single mutants produce less ammonia during the second ammonia pulse than colonies of the parental strain BY4742 (Figure 3B). Volatile ammonia release can be enhanced under conditions of higher pH of a colony surroundings and consequent ammonium deprotonation. To exclude the possibility that the absence of Ycr010p, Ydr384p, and Ynr002p proteins influences the extracellular pH rather than ammonia production, we picked up the cells from colonies occurring in distinct developmental phases, and we compared their ability to produce ammonium/ammonia under conditions of identical pH. Figure 4E shows that cells taken from phase 6 alkali colonies (strongly expressing *YCR010c*, *YDR384c,* and *YNR002c* genes) produce higher amount of ammonium/ammonia during 25-min incubation in MES buffer of pH 6 than cells taken from phase 2 acid colonies. The cells from all three mutated alkali colonies (*ycr010c*, *ydr384c,* and *ynr002c*) produced significantly lower amounts of ammonium/ammonia than cells from alkali colonies of the isogenic parental strain (Figure 4E). These observations exclude the possibility that the defect of ammonia production by colonies of individual *ycr010c*, *ydr384c,* and *ynr002c* mutants is only due to the differences in external pH.

To analyze whether changes of external pH influence the level of ammonium/ammonia production, we analyzed the kinetics of ammonium/ammonia production by cells from alkali colonies of parental strain in buffers of pH range from 4–7. After the first 10 min of incubation at pH 4.0, the ammonium production by cells from alkali colonies was greatly potentiated when compared with that by cells incubated at pH 7.0 (Figure 4F). This effect could be coherent with proton import coupled to ammonium production, which can be limiting at pH 7.0 but not at pH 4.0.

Then, we compared the methyl ammonium (toxic analogue of ammonium) tolerance of cells from colonies in the acid phase, with that of cells from colonies in the alkali phase. The cells were treated for 1 h in buffer of pH 6 with 0–100 mM methyl ammonium. The surviving cells were estimated as the number of colony forming units. The cells from alkali colonies survived treatment with ~5 times higher concentration of methyl ammonium than cells from acid colonies (Figure 9S at http://www.biologie.ens.fr/fr/genetiqu/puces/publications/ATO/index.html). This result indicates that cells strongly expressing the *YCR010c*, *YDR384c,* and *YNR002c* genes might transport methyl ammonium outwards (against its gradient) more efficiently than cells weakly expressing these genes, thus decreasing its toxic effect. However, also other explanations could be imagined.

All these observations support an importance of Ycr010p, Ynr002p, and Ydr384p in Ammonia (Ammonium) Transport Outward of *S. cerevisiae* colonies. We, therefore, named their genes *ATO1, ATO2,* and *ATO3*, respectively.

Plasma Membrane Transporters

In the early phases (between 1 and 2), three genes encoding plasma membrane permeases (*YLR004c*, *YOL119c*, *YOR306c*), which are probably involved in carboxylic acid transport (Paulsen *et al.*, 1998), were activated. This might indicate that the uptake of carboxylic acids is activated in parallel with the induction of amino acid metabolism. Thereafter (during phases 2–4), carboxylic acids could be metabolized by enzymes involved in glyoxylate and dicarboxylate pathways.

The expression of several genes encoding plasma membrane permeases transporting various ions and possibly influencing the pH or electric polarity of the plasma membrane was also altered. Genes encoding transporters of sulfate (*SUL1*, *SUL2*) and zinc (*ZRT1*) were activated early, between phases 1 and 2. Genes encoding transporters of phosphate (*PHO84*, *PHO89*) were activated one phase later (between 2 and 3). From the time of the first detectable ammonia production (phase 4) the expression of *PMA1* and *PMA2* genes encoding plasma membrane H^+ ATPases gradually declined. This should decrease the ability of cells to extrude protons and to decrease the extracellular pH.

Early Repression of Oxidative Mitochondrial Functions and Later Changes in the Citrate Cycle

Repression of several genes functionally connected to mitochondrial oxidative phosphorylation and energy generation starts during the phases 1–3 and gradually intensifies during phases 5 and 6, where it is most pronounced (Figure 2). Genes encoding enzymes belonging to the electron transport system of complex I (*NDI1*), complex II (*SDH1*, *YJL045w*), complex III (*QCR2*, *QCR7*), complex IV (*COX4*, *COX5A*, *COX9*, *COX13*) and ATP synthase (*ATP2*, *ATP15*) together with the mitochondrial membrane phosphate transporters (*MIR1*, *YER053C*), a protein involved in the import of mitochondrial matrix proteins (*TIM17*) and the outer mitochondrial membrane porin (*POR1*), which is important for NADH flux, were repressed. This indicates a generalized decrease in respiratory mitochondrial functions. Slightly later (between phases 4 and 5), genes encoding enzymes of

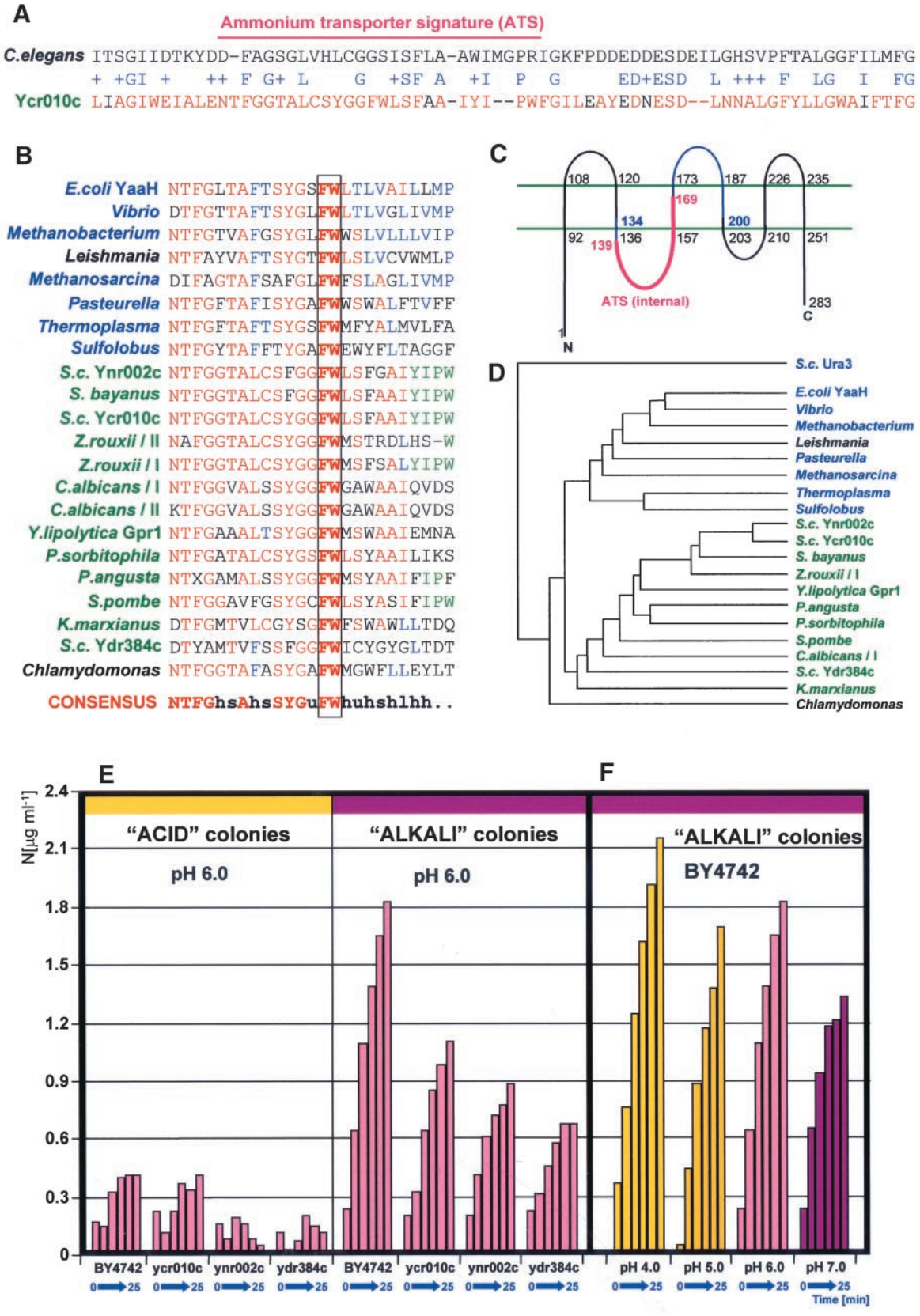
A
Ammonium transporter signature (ATS)
C.elegans ITSGIIDTKYDD-FAGSGLVHLCGGSISFLA-AWIMGPRIGKFPDDEDDESDEILGHSVPFTALGGFILMFG
+ +GI + ++ F G+ L G +SF A +I P G ED+ESD L +++ F LG I FG
Ycr010c LIAGIWEIALENTFGGTALCSYGGFWLSFAA-IYI--PWFGILEAYEDNESD--LNNALGFYLLGWAIFTFG
B
E.coli YaaH NTFGLTAFTSYGSFWLTLVAILLMP
Vibrio DTFGTTAFTSYGLFWLTLVGLIVMP
Methanobacterium NTFGTVAFGSYGLFWWSLVLLLVIP
Leishmania NTFAYVAFTSYGTFWLSLVCVWMLP
Methanosarcina DIFAGTAFSAFGLFWFSLAGLIVMP
Pasteurella NTFGFTAFISYGAFWWSWALFTVFF
Thermoplasma NTFGFTAFTSYGSFWMFYALMVLFA
Sulfolobus NTFGYTAFFTYGAFWEWYFLTAGGF
S.c. Ynr002c NTFGGTALCSFGGFWLSFGAIYIPW
S. bayanus NTFGGTALCSFGGFWLSFAAIYIPW
S.c. Ycr010c NTFGGTALCSYGGFWLSFAAIYIPW
Z.rouxii / II NAFGGTALCSYGGFWMSTRDLHS-W
Z.rouxii / I NTFGGTALCSYGGFWMSFSALYIPW
C.albicans / I NTFGGVALSSYGGFWGAWAAIQVDS
C.albicans / II KTFGGVALSSYGGFWGAWAAIQVDS
Y.lipolytica Gpr1 NTFGAAALTSYGGFWMSWAAIEMNA
P.sorbitophila NTFGATALCSYGSFWLSYAAILIKS
P.angusta NTXGAMALSSYGGFWMSYAAIFIPF
S.pombe NTFGGAVFGSYGCFWLSYASIFIPW
K.marxianus DTFGMTVLCGYSGFWFSWAWLLTDQ
S.c. Ydr384c DTYAMTVFSSFGGFWICYGYGLTDT
Chlamydomonas NTFGGTAFASYGAFWMGWFLLEYLT
CONSENSUS NTFGhsAhsSYGuFWhuhshlhh..
C
108 120 173 187 226 235
169
134 200
92 139 136 157 203 210 251
ATS (internal)
283
1 N
C
D
S.c. Ura3
E.coli YaaH
Vibrio
Methanobacterium
Leishmania
Pasteurella
Methanosarcina
Thermoplasma
Sulfolobus
S.c. Ynr002c
S.c. Ycr010c
S. bayanus
Z.rouxii / I
Y.lipolytica Gpr1
P.angusta
P.sorbitophila
S.pombe
C.albicans / I
S.c. Ydr384c
K.marxianus
Chlamydomonas
E
F
N[µg ml-1]
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
"ACID" colonies
pH 6.0
"ALKALI" colonies
pH 6.0
"ALKALI" colonies
BY4742
BY4742 ycr010c ynr002c ydr384c BY4742 ycr010c ynr002c ydr384c pH 4.0 pH 5.0 pH 6.0 pH 7.0
0→25
Time [min]

Figure 4.

the mitochondrial citrate cycle (*ACO1*, *MDH1*, *IDH1*, *IDH2*, *FUM1*) became significantly repressed. In parallel, the genes encoding ADP/ATP carrier proteins (Pet9p, Aac3p) and the dicarboxylate transporters Odc1p and Sfc1p were repressed.

All these events indicate a progressive decrease in mitochondrial activity during the acid/alkali transition. One exception was the induction of the *OAC1* gene, encoding a mitochondrial transporter probably involved in oxaloacetate transport from the cytoplasm into mitochondria (Palmieri *et al.*, 1999). The *OAC1* gene is activated early, between phases 1 and 2, simultaneously with the activation of amino acid metabolic genes, and peaked between phases 3 and 4, when the only induced enzyme of the mitochondrial citrate cycle genes, *CIT3* (encoding citrate synthetase which uses oxaloacetate and AcetylCoA as the substrates for citrate and CoA production), became strongly activated. These findings indicate that oxaloacetate is transported from the cytoplasm into mitochondria where it is converted into citrate, which probably cannot efficiently enter the citrate cycle in later phases 5–6. An enzyme that might be involved in further citrate conversion in mitochondria, 2-methylisocitrate lyase (converting citrate to succinate and glyoxylate), is encoded by *ICL2*, which was induced at the same time as *CIT3*. Alternatively, the mitochondrial oxaloacetate might be used by Aat1p for amino acid biosynthesis. Other genes that were activated in the same period include *ALD4* and *ALD5* encoding two mitochondrial aldehyde dehydrogenases catalyzing the oxidation of acetaldehyde to acetate and thus regenerating NADH.

Peroxisome Function and Fatty Acid β-Oxidation

Genes encoding enzymes involved in fatty acid β-oxidation and acetylCoA production in peroxisomes (*POX1*, *FOX2*, *POT1*, *ECI1*, *DCI1*, *IDP3*, *CTA1*, *ACS1*, *FAA2*), in the transport of fatty acids across the peroxisomal membrane (*PXA1*, *PXA2*) and in peroxisome biogenesis and targeting (*PEX11*, *YMR018w*) were activated during the interval of ~12 h between phases 3 and 4 (Figure 2). This indicates that at the beginning of detectable ammonia production, the fatty acid stocks of cells in colonies are mobilized and converted into acetyl-CoA in peroxisomes. The *CAT2* gene for carnitine *O*-acetyltransferase, involved in acetyl-CoA transport from peroxisomes to the mitochondria (van Roermund *et al.*, 1999), is activated in the same phase, indicating that acetylCoA is transported into the mitochondria where it can be used by citrate synthase Cit3p (Figures 5 and 6).

"Stress" Genes and Adaptation

The comparison of genes found to be induced and repressed in our experiments with the database of genes induced or repressed under various external stress conditions (Gasch *et al.*, 2000; Causton *et al.*, 2001) revealed interesting differences between our induced and our repressed genes. The genes repressed in the later alkali phase (i.e., genes that were more highly expressed in phase-1 acid colonies) include several genes classified in the environmental stress response (ESR) functional family (e.g., *CTT1*, *YGP1*, *HSP30*, Figure 2; Gasch *et al.*, 2000). Similarly, the *MSN4* gene, encoding a transcription factor activating several ESR genes (Causton *et al.*, 2001), was repressed during the acid/alkali transition. In contrast, the group of the genes induced in alkali colonies, does not include ESR genes, whereas it includes a lot of genes that are strongly activated in the middle/late stationary phase (e.g., *ATO1,3*, *PHO89*, several fatty acid metabolism genes and peroxisome biogenesis genes, *CIT3*, *ICL2*, *CAT2*; Gasch *et al.*, 2000).

In contrast to the action of different drugs that are not common in nature and, therefore, induce an immediate "stress response," cells in natural conditions often face limited nutrient supply. In such cases, their ability to enter the stationary phase (connected with their increased resistance to starvation) is the only mechanism except sporulation that enables them to survive. This implicates the necessity of an active mechanism for adaptation, both at the entry into the stationary phase and also later, for maintenance of either slow "stationary growth" or long-term survival. The transition of yeast colonies from acid to alkali phase might be part of this kind of adaptive mechanism. The progressive repression of ESR genes might, therefore, be perceived as an indication of an "escape" from the stress-like conditions prevalent in acid developmental phase and a successful adaptation of alkali colonies to starvation (Figure 5). This scenario is supported by the behavior of colonies of the *sok2* mutant. The *SOK2* gene was 2.7-fold induced in alkali colonies of our, as yet uncharacterized, *S. cerevisiae* mutant overproducing ammonia (our unpublished data). *SOK2* encodes a transcription factor negatively regulating filamentous growth (Ward *et al.*, 1995; Pan and Heitman, 2000). The growth rate of *sok2* colonies did not differ from that of the parental strain until they reached the beginning of the alkali phase. At this stage the colonies were not able to reach the intense ammonia production (Figure 3B). They stopped growing, their morphology became irregular, and several papillae formed by cells, which apparently did not share the fate of the majority and probably cannibalize on dying cells, appeared on the colony surface (Figure 3C). Papillae were observed previously on colonies of *S. cerevisiae shr3*, which

Figure 4 (facing page). *S. cerevisiae* members of YaaH family are involved in ammonia production in colonies. (A) Region of homology of Ycr010p with the *C. elegans* putative ammonium transporter, P54145. Blue letters indicate the similarity. (B) The regions of similarity of YaaH family proteins with the putative ATS of Ycr010p. Most conservative amino acids (red). Nonconserved amino acids (black). Yeast sp. and amino acids conserved among yeast genes (green). Bacterial sp. and sequences conserved among bacterial genes (blue). The accession numbers of individual proteins are on Figure 4 at www.biologie.ens.fr/yeast-publi.html. The consensus sequence is indicated below, h, hydrophobic amino acids (A, C, F, G, H, I, K, L, M, R, T, V, W, Y), s, small amino acids (A, C, D, G, N, P, S, T, V), u, tiny amino acids (A, G, S), and l, aliphatic amino acids (I, L, V). (C) TMHMM prediction of the transmembrane localization of Ycr010p. Numbers indicate amino acids. Blue, region of homology with *C. elegans* P54145; violet, region of homology with ammonium transporter signature (ATS). (D) Average distance tree of YaaH family proteins. Ura3p of *S. cerevisiae* was used as a nonrelated protein control. (E) Ammonium production by cells from acid or alkali colonies of BY4742 and mutants *ycr010c*, *ydr384c,* and *ynr002c*. Samples of cells suspended in 10 mM MES (pH 6.0) were taken at 0, 5, 10, 15, 20, and 25 min of incubation. (F) Ammonium production by BY4742 cells from alkali colonies at different pH values. Samples of cells suspended in 10 mM MES of different pH (4, 5, 6, or 7) were taken at 0, 5, 10, 15, 20, and 25 min of incubation.

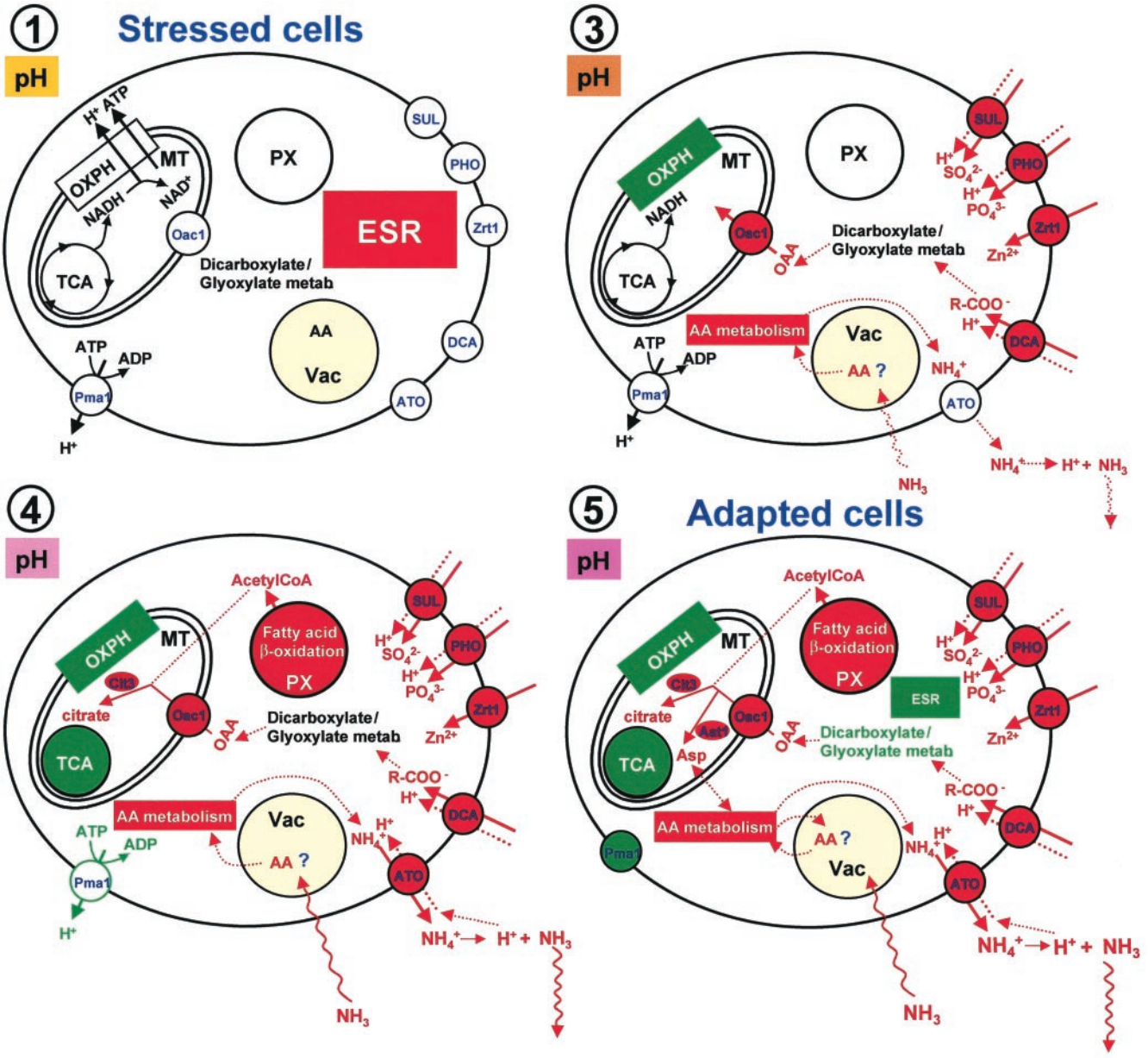

Figure 5. Model of metabolic changes within *S. cerevisiae* colonies during the acid/alkali transition. Summary of the important changes occurring in colonies in phases 1, 3, 4, and 5. A detailed description of the individual processes is given in the results and in Figure 2. The proposed relationships between the processes are described in the DISCUSSION. Red color indicates the activated expression of a particular functional gene group, green color indicates the repression. MT, mitochondria; PX, peroxisome; V, vacuole; OXPH, oxidative phosphorylation system; TCA, enzymes of tricarboxylate (citrate) cycle; ESR, environmental stress response factors; SUL, transporters of sulfate; PHO, transporters of phosphate; DCA, transporters of carboxylic acids; ATO, yeast members of YaaH family; AA, amino acids; OAA, oxaloacetate.

has a defect in proper localization of several amino acid permeases (Ljungdahl *et al.*, 1992) and is also defective in ammonia production and in acid/alkali transition (Palková *et al.*, 1997). In acid *sok2* colonies, the *MSN4* gene, the product of which activates several ESR genes, was expressed several-fold more than in colonies of wild-type parental strain (Figure 3D).

DISCUSSION

Regulation of the Transition of Colonies from Acid to Alkali Phase

The observed metabolic changes and the "behavior" of specific mutants indicated the existence of at least two particu-

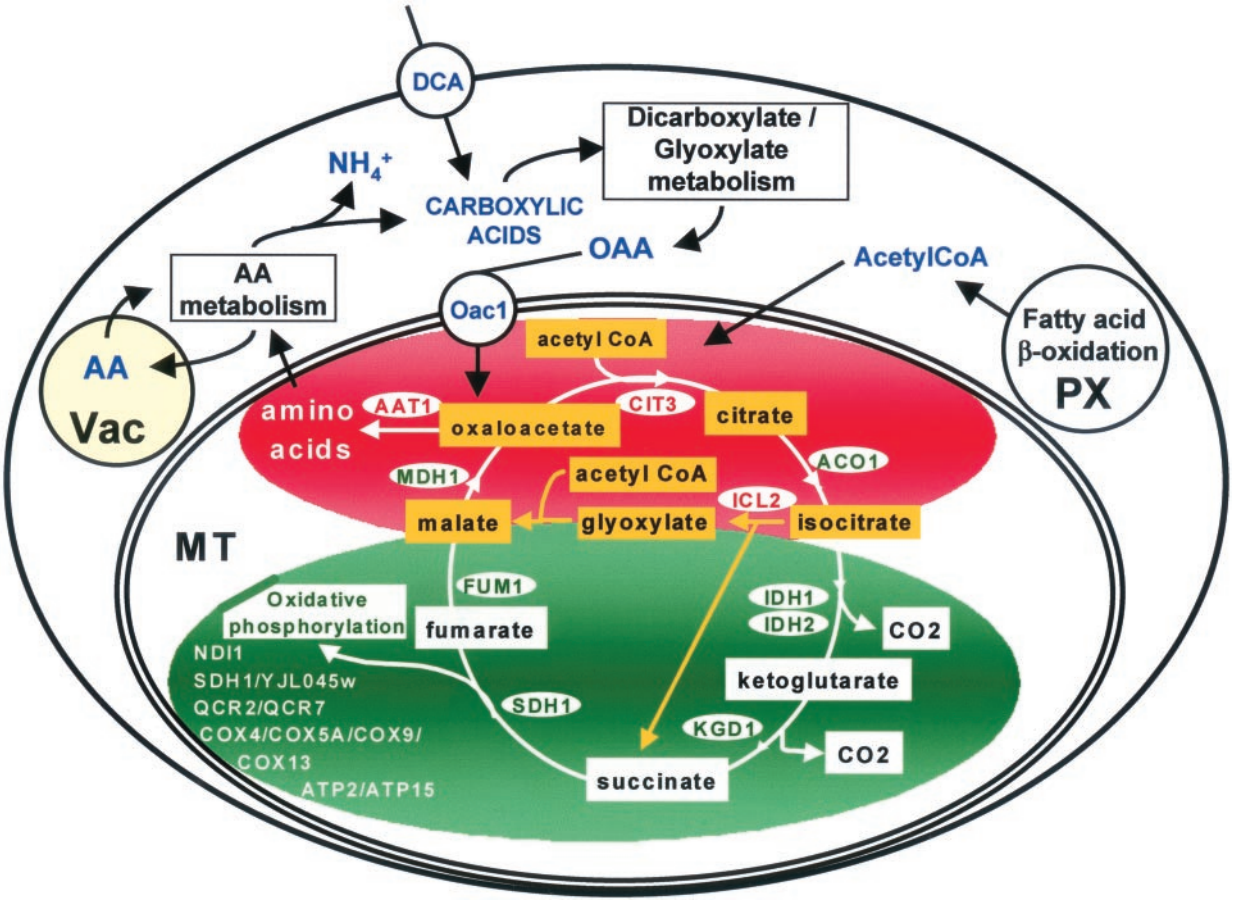

Figure 6. Model of the yeast glyoxylate bypass pathway and its possible connection with other predicted changes. Red part indicates the activated glyoxylate bypass. Green part indicates repressed part of the TCA cycle and oxidative phosphorylation. Notice that the limiting enzymes of glyoxylate bypass and oxaloacetate turn over are Cit3 and Icl2 (activated in phase 5) and Idh1/Idh2 (repressed in phase 5). The expression of ACO1 gene is twofold repressed in phase 5, but this enzyme seems to be very highly expressed in the cells (our own measurements), and this repression may not be detrimental to the glyoxylate bypass efficiency. The gene expression pattern suggests a switch from TCA degradative cycle to glyoxylate bypass at phase 5 of the acid/alkali transition.

lar checkpoints during the transition process. First, changes connected with the "decision" of colonies to proceed from the acid phase to the phase of the moderate ammonia production and second, changes that result in the induction and enhancement of ammonia release. Factual intracellular signals and transcription regulators involved in both checkpoints have still to be determined. The activation of several genes connected with amino acid metabolism between phases 1 and 2 (Figure 2), the transient changes in intracellular amino acid concentrations (Figure 1, C and D) and the connection between the uptake of external amino acids and the ability of colonies to produce ammonia (Palková *et al.*, 1997; Zikánová *et al.*, 2002) indicate that amino acid depletion might be important for the primary decision of colonies to proceed to the alkali period of life. Most of the genes from the "amino acid" group are regulated by the transcription factor Gcn4p when amino acids are limiting (Rolfes and Hinnebusch, 1993) or contain a Gcn4-binding consensus sequence in their promoter (Figure 10S at http://www.biologie.ens.fr/fr/genetiqu/puces/publications/ATO/index.html). The gene encoding the transcription meiotic regulator Ime1p was induced between phases 1 and 2, and its induction gradually increased until phase 5 (Figure 2). Recently, it was shown that Ime1p associates with the N-terminal domain of Sok2p in the absence of glucose, converting it to an activator (Shenhar and Kassir, 2001). This, together with indications that the same Sok2p domain might also bind the Msn2p transcription factor (Shenhar and Kassir, 2001), implies that Ime1p might be involved in changing the Sok2p function during the acid/alkali transition.

Model of Ammonia Release in Colonies

Activation of various amino acid catabolic enzymes (Figure 2) and observed transient decrease of amino acid vacuolar pool at the beginning of ammonia production (Figure 1D) indicate that amino acids released from vacuoles might be a source of NH_3/NH_4^+ in the cells. Until now, we have no evidence whether alkali colonies directly produce unprotonated ammonia (which might diffuse through membranes

and therefore does not require a transport protein) or whether they actively export protonated ammonium (NH_4^+) and, in parallel, increase the external pH (possibly by driving protons into cells). In both situations, the extracellular NH_3/NH_4^+ ratio would be increased and volatile NH_3 would spread in the surroundings. Because NH_3 is toxic in high concentrations, it seems unlikely that it is freely present within the cells. Genes for several permeases, which might be involved in pH changes, are induced before the detectable ammonia production started (Figures 2 and 5). Pho84p was previously shown to function as a phosphate/H^+ symporter (Persson *et al.*, 1998), thus decreasing the level of extracellular H^+. The protonated forms of carboxylic acids may be transported into cells by the Ylr004p, Yol119p, and Yor306p permeases. Early activation of various transporters, which might cotransport protons into the cells, together with the gradual repression of genes encoding plasma membrane H^+ ATPases (extruding protons from the cells), may contribute to the increase in extracellular pH, acidification of the cytoplasm, and thus depolarization of the plasma membrane.

The *S. cerevisiae* members of YaaH family (Ato proteins) seem to be important for ammonia production by *S. cerevisiae* colonies (see also Figures 2–4). They have six predicted transmembrane spans. They contain a region of homology with an established ATS. Their genes are strongly expressed during the alkali phase of colony growth that is accompanied by increased ammonia production. Their deletion decreases ammonium/ammonia production both in colonies and also by cells suspended in the buffer. Ammonium/ammonia production is more pronounced at pH 4.0 than at pH 7.0 within the time range of 10–25 min of incubation. The cells highly expressing ATO genes are more resistant to the toxic effect of methyl ammonium. All these results are compatible with the simple interpretation that *S. cerevisiae* Ato proteins act as ammonium/H^+ antiporters, extruding ammonium from the yeast cells and importing protons. Ato proteins may thus contribute also to the increase of external pH and consequent ammonia release from NH_4^+ (Figure 5). However, it cannot be excluded that Ato proteins are not directly pumping ammonium/ammonia out of the cells of alkali colonies. They can be involved either in a transport of other, yet unidentified substrates, or in a transfer of a regulatory signal. Subsequently, both could enhance somehow the ability of cells of alkali colonies to release ammonia to the surroundings.

Model of Metabolic Alterations and Stress Adaptation of Colonies during the Acid/Alkali Transition

Considering gene expression alterations, the following metabolic and transport changes can be expected (Figures 5 and 6). Sequentially, activation of amino acid catabolism, uptake of carboxylic acids into cytoplasm, uptake of oxalacetate into mitochondria, progressive repression of oxidative phosphorylation, peroxisomal fatty-acid β-oxidation leading to acetylCoA production, citrate and isocitrate synthesis in mitochondria and repression of other enzymes of the citrate cycle (Figure 5). Some of these events remind the metabolic state of bacteria growing on carboxylic acids (acetate) or fatty acids as sole carbon sources, known as glyoxylate bypass (Cozzone, 1998). Under such conditions, bacterial cells bypass the degradative steps of the citrate cycle (connected with CO_2 production), while maintaining oxaloacetate turnover important for the biosynthesis of "complex" molecules like amino acids and sugars. In simplified form: AcetylCoA (from acetate or fatty acids) and oxaloacetate are turned to isocitrate, which is converted to glyoxylate and succinate by isocitrate lyase, the key enzyme of this pathway. The cycle is completed by resynthesis of oxaloacetate from glyoxylate and acetylCoA. The glyoxylate bypass is the only pathway enabling growth of microorganisms on acetylCoA. (Cozzone, 1998)

In yeast colonies, a role of metabolic pathway analogous to bacterial glyoxylate bypass appears to be possible (Figure 6). First, at the beginning of acid/alkali transition, carboxylic acids can be both imported from surroundings via carboxylic acid transporters and also produced during amino acid deamination, a process that seems to be important for ammonia release. The latter is supported by observed decrease in intracellular amino acid concentration at the beginning of ammonia production (Figure 1C). The indication that carboxylic skeletons are subsequently utilized to oxaloacetate and acetylCoA rather then removed again from cells comes out from observed increase of extracellular pH. The *Oac1* carrier could be involved in transport of oxalacetate from cytosol to mitochondria. Second, the later activation of fatty acid β-oxidation leading to further production of acetylCoA temporally synchronizes with the peak of gene activation of mitochondrial citrate synthase (Cit3p) and isocitrate lyase (Icl2p). The parallel activation of the gene of mitochondrial aspartate aminotransferase (AAT1) indicates the connection of oxaloacetate to amino acid biosynthesis, which might be important for observed recovery of vacuolar amino acid stocks. In contrast to observed changes indicating a role of mitochondrial glyoxylate cycle, the genes of cytosolic glyoxylate enzymes were repressed at the end of the estimated period. Nevertheless, because we have no data from earlier times of acidic phase, we cannot exclude their previous activation.

Recently, it was shown that there might be a link between mitochondrial dysfunction and the activation of peroxisomes and fatty acid metabolism in rho° and antimycin-treated cells growing in liquid raffinose media (Epstein *et al.*, 2001). Likewise, in colonies, the decline of "mitochondrial" oxidative phosphorylation, starting during the early phases, was followed with later rapid increase of peroxisomal function (Figures 2 and 5).

The observed metabolic transition seems to be critical for long-term yeast colony development. The changes are accompanied by a significant decrease in the expression of several genes involved in ESR and also of the gene encoding ESR activator Msn4p. This suggests the cumulation of stress conditions in late acid phase colonies and their elimination during the alkali phase transition. Such stress conditions are possibly caused by amino acid starvation and oxidative stress evoked by mitochondria function. In these regards, the cyclic acid/alkali oscillation of yeast colonies might be interpreted as switches from growth period of energy-producing metabolism finally associated with increase of oxidative radicals and nutrient depletion to a period of transient growth arrest of alternated metabolism finally resulting in reconstitution of bio-molecule stocks (see amino

acids in Figure 1D) and stress reduction. The possible connection between the acid/alkali transition and ammonia release and long-term colony surviving is supported also by the behavior of colonies of *sok2* and *shr3* mutants, which both are unable to accomplish the transition to the alkali phase and do not produce ammonia. Instead of it they stop to grow and cover by papillae formed by cells probably cannibalizing on dying ones (Figure 3C; Palková *et al*., 1997). Moreover, the sok2 colonies contain several-fold higher level of expression of MSN4 in acid phase when compared with colonies of parental strain (Figure 3D).

Thus, the ammonia release might function as an alarm signal produced by a colony, the cells of which first detected limited nutrients. The ability of volatile ammonia to induce strong ammonia production in the neighboring colonies (and thus to elicit the alkali phase) might spread this alarm throughout the whole population, which adapts efficiently to limited nutrient conditions before the various stresses exceed a detrimental level. Examples of similar "behavior" can be found among different microorganisms. For example, in bacterial populations of *Streptomyces griseus* growing on solid surfaces, the first cells, which detect nutrient depletion start to produce specific pheromone. The pheromone subsequently induces its own production by neighboring bacteria, and the signal is spread throughout the whole population, the metabolism of which is altered and this initiates the formation of aerial hyphae and sporulation (Dunny and Leonard, 1997). The periodicity of the pH changes around yeast colonies and, therefore, the ability of colonies reaching the alkali phase to resume growth and consequently to enter the next acid phase implies that the alkali developmental stage is not the terminal one (Palková and Forstová, 2000).

Ammonia Signaling: The Perspectives

The postulated role of volatile ammonia in the ability of "stationary" yeast cells to change their metabolism, escape from the previous stressful conditions, and consequently survive for long periods, might be important also in higher eukaryotes where ammonia/ammonium is an important signaling molecule for long-lived neurons. Ammonia/ammonium exhibits a variety of biochemical and neurological effects and when present in excess it can disturb reversible reactions causing "hyperammonemiac" symptoms connected with several serious diseases (Butterworth, 1998).

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